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SPECIAL ISSUE: EXTRACELLULAR RNA COMMUNICATION CONSORTIUM

Potential functional applications of extracellular vesicles: a report by the NIH Common Fund Extracellular RNA Communication Consortium

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The NIH Extracellular RNA Communication Program's initiative on clinical utility of extracellular RNAs and therapeutic agents and developing scalable technologies is reviewed here. Background information and details of the projects are presented. The work has focused on modulation of target cell fate by extracellular vesicles (EVs) and RNA. Work on plant-derived vesicles is of intense interest, and non-mammalian sources of vesicles may represent a very promising source for different therapeutic approaches. Retro-viral-like particles are intriguing. Clearly, EVs share pathways with the assembly machinery of several other viruses, including human endogenous retrovirals (HERVs), and this convergence may explain the observation of viral-like particles containing viral proteins and nucleic acid in EVs. Dramatic effect on regeneration of damaged bone marrow, renal, pulmonary and cardiovascular tissue is demonstrated and discussed. These studies show restoration of injured cell function and the importance of heterogeneity of different vesicle populations. The potential for neural regeneration is explored, and the capacity to promote and reverse neoplasia by EV exposure is described. The tremendous clinical potential of EVs underlies many of these projects, and the importance of regulatory issues and the necessity of general manufacturing production (GMP) studies for eventual clinical trials are emphasized. Clinical trials are already being pursued and should expand dramatically in the near future.

Keywords: *extracellular vesicles; cell fate change; functional effects; renal; pulmonary heart disease; cancer*

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The NIH Extracellular RNA Communication Program has 5 key initiatives: 1) General reference catalogue of extracellular RNAs (exRNA) in bodily fluids of normal individuals. 2) Defining fundamental principles of exRNA biogenesis, distribution, uptake and function and development of molecular tools technologies

and imaging modalities to enable these studies. 3) Identification of exRNA biomarkers. 4) Demonstrating the clinical utility of exRNAs as therapeutic agents and developing scalable technologies. 5) Developing a community resource, the exRNA atlas, to provide access to exRNA data, standardized exRNA protocols and other useful tools.

This contribution summarizes work on the 4th initiative on functional effects, clinical utility and to some extent biomarkers.

The definition of the functional effects and clinical potential of extracellular vesicles is a rapidly expanding field. Vesicle effects have now been shown in multiple domains of cell biology and clinical disease. Vesicles have in general been considered as either exosomes or micro-vesicles (1), the first originating from the endosomal compartment and the latter from the cell membrane. They were originally defined by size and morphology, but given significant overlap, it has been suggested that they simply be termed extracellular vesicles (EVs) (2). They contain proteins, genomic and mitochondrial DNA, lipids, mRNA and non-coding RNA and can deliver these entities to target cells resulting in changes in cell phenotype or repair of cell injury (3–5). Their contents and surface characteristics vary markedly with the nature of the originating cell, the treatment of the originating cells and the isolation techniques employed, and their biologic effects vary tremendously with the nature of the impacted target cell; thus, a prime characteristic is their heterogeneity. The problems with strict identification of EVs has been highlighted by a consensus article from the International Society of Extracellular Vesicles which also put forward some evolving criteria for indicating that investigators were working with vesicles (6). A number of descriptions of changes in cell phenotype after vesicle exposure have been reported, but it is important to realize that the nature of the vesicle and its biologic effects are very context dependent.

It is of note that thus far three phase 1 clinical immunotherapy trials reporting feasibility and safety of autologous EV therapeutics have been published (7,8), and 4 additional EV-based clinical trials have been registered at ClinicalTrials.gov. Regulatory criteria for EV-based therapies are still in evolution.

Extracellular vesicles in therapy and regenerative medicine

A major interest has been the capacity of EVs to restore injured tissue in regenerative medicine. Several projects in the Extracellular RNA Communication Consortium subgroup focused on exRNA therapy are focused on demonstrating therapeutic potential of exRNAs and to develop methods and tools to enable targeting specific cells in attempts to repair injured tissue.

Plant vesicles

The project of Dr. Zhang and colleagues was to evaluate exosome-like nanoparticles for therapeutic delivery of RNAs. The existence and role of plant vesicles has been generally under appreciated. Plant cells release vesicles that contain proteins, lipids and RNAs (9,10). Their size distribution ranges from 100 to 400 nm in diameter with a negative zeta potential ranging from -49.2 to -1.52 mV;

their content varies with origin. These exosome-like nanoparticles are highly resistant to gastric pepsin solution and to intestinal pancreatic and bile extract solutions, thus indicating their potential for influencing cell biology through ingested foods (10,11). Edible plant exosome-like nanoparticles can be taken up by intestinal macrophages and stem cells, thus preventing dextran sulphate sodium-induced mouse colitis (9,10). These effects may be crucial for maintaining intestinal homeostasis and gut immune tolerance. The specific project here is to develop fruit exosome-like vectors for multi-modal strategies to deliver miRNA in a tissue-specific manner. Developing therapeutic agents must consider issues of targeting delivery, safety and the cost of large-scale production. Dr Zhang has developed a grapefruit-derived nano-vector to meet all these requirements for safety, targeting and delivery (12,13). Recently, the data generated from clinical trials also support the concept that oral administration of grape-exosome-like nanoparticle is potentially an effective and safe treatment that may prevent the progression of head/neck cancer (this data not published, but web link for the trial is www.clinicaltrials.gov/ct2/show/NCT01668849).

Reversal of renal damage by mesenchymal stem cell-derived vesicles

Mesenchymal stem cells (MSCs) are multipotent adult stem cells which have immunomodulatory properties (14), the capacity to differentiate into multilineage cells of mesenchymal origin (15) and to migrate to the site of an injury (16). MSCs have been shown to have reparative effects in many settings. Clinical trials involving MSCs include cardiovascular diseases, bone and cartilage defects, spinal cord injury, graft-versus-host disease, Crohn's disease, diabetes and acute kidney injury (17). MSCs can have curative effects in models of kidney injury and detailed studies indicate that vesicles from the bone marrow-derived MSCs mediate the observed healing effects (18). The bone marrow-derived MSC EVs in these studies were in the nano-range (60–170 nm) and expressed specific markers of mesenchymal lineage (CD105, CD73, CD44 and CD29) and of exosomes (LAMP-1, CD63). MSC-derived EVs intravenously injected into mice selectively localized to injured kidneys (19). Further experiments on CD29 integrin and CD44 blockade indicated that they were involved in vesicle uptake by tubular cells (18). The MSC-derived vesicles translocated to transcription regulator tubular epithelial cells, which promoted downregulation of genes involved in cell apoptosis (CASP1, CASP8 and LTA) and upregulation of anti-apoptotic genes (BCL-XL, BCL2, and BIRC8) (16) indicating possible healing mechanisms for the injured renal tissue. In addition, in a model of cisplatin-injured murine renal tubular cells, MSC-derived EVs were shown to facilitate the transfer of IGF-1R mRNA and to induce expression of human IGF-1R (20). This factor

was shown to promote tubular cell proliferation by increasing cell sensitivity to IGF-1. Human umbilical MSC-derived EVs were also shown to be beneficial in cisplatin-induced acute kidney injury by activation of the extracellular-signal-regulated kinase (ERK) 1/2 pathway, which is involved in tubular cell proliferation and protection from apoptosis (21). Moreover, in a chronic mouse model of renal disease EVs released by MSCs were shown to prevent fibrosis (22). Based upon studies of RNase sensitivity and on the effects of Droscha knock-down in vesicle originator cells (23), it was suggested that the observed healing effects were mediated by miRNA species. Elucidation of the miRNA targets is ongoing. In vitro studies on renal tubular epithelial cells exposed to ischemia-reperfusion injury induced by ATP depletion showed a modulation of miRNA expression by MSC-derived EVs partly due to miRNA transfer via EVs, and partly to EV-triggered transcription (24).

Pulmonary hypertension and extracellular vesicles

Pulmonary hypertension is a syndrome characterized by vascular remodelling and right ventricular hypertrophy and is associated with many different diseases. It is treatable but not curable and it is eventually fatal. Its aetiology is unknown. Aliotta and colleagues have been evaluating a murine model of pulmonary hypertension: the monocrotaline-treated mouse. They have found that vesicles isolated from the plasma or lungs of these mice induce pulmonary hypertension when infused into normal mice (25). Moreover, bone marrow cells from the hypertensive mouse when infused into irradiated mice induced pulmonary hypertension. Most recently, they have shown that lung-derived vesicles traffic to bone marrow induce “toxic” endothelial progenitors, which then have the capacity to induce pulmonary hypertension in normal mice. Thus, we have initial insights into cellular mechanisms underlying this disease.

In separate studies, the Aliotta group has found that vesicles from bone marrow-derived MSCs can both prevent and reverse the development of pulmonary hypertension in the monocrotaline mouse model. This suggests that MSC vesicles may have a role in treating pulmonary hypertension. Further studies suggest that the “good” vesicles from bone marrow-derived MSCs alter the phenotype of the “toxic” vesicles back to normal.

MSC reversal of radiation-induced marrow stem cell injury

Radiation exposure results in different levels of tissue injury depending on dose, including the immune system, the hematopoietic system, gastrointestinal tract, kidney, skin and lung (26,27). Hematopoietic cells are sensitive to radiation and exposure can result in bone marrow failure and potentially lethal haemorrhage or infections. Six

months after exposure to 100cGy whole-body irradiation, the engraftment capacity of murine marrow is reduced to 20% of the non-irradiated control bone marrow (28). A number of radiation mitigators have been described which improve hematopoietic recovery from irradiation damage. The transplantation of bone marrow can restore haematopoiesis in lethally irradiated subjects (29–31). However, aside from transplantation, the efficacy of these treatments is relatively limited and temporally constrained.

The MSC is a cell population with multipotential and plays a critical role in microenvironmental support of the hematopoietic stem cell (15,32,33). The capacity of MSCs for tissue repair has been reported in past decades. The protective mechanisms are believed to be related to either their differentiation capacity or to paracrine effects (34,35). Transplantation of MSCs alone or with hematopoietic stem cells has also been shown to enhance engraftment and improve bone marrow recovery from radiation injury (36–41).

Recent studies have shown that MSC-derived vesicles mediate reversal of different tissue injuries to kidney, brain and myocardium (18,22,42–44). In this study, Quesenberry and colleagues evaluated whether bone marrow MSC-derived vesicles could reverse irradiation damage to bone marrow stem/progenitor cells.

In our project, we have evaluated the capacity of bone marrow-derived MSC vesicles to mitigate radiation injury to bone marrow stem cells at 4 hours to 7 days after irradiation. Significant restoration of bone marrow cell engraftment at 4, 24 and 168 hours post-irradiation by exposure to MSC-EV was evaluated at 3 weeks to 9 months after transplant and further confirmed by secondary engraftment. The Quesenberry group demonstrated the recovery of peripheral blood counts and restoration of the engraftment of bone marrow by intravenously injected MSC-EV to 500 cGy exposed mice. Moreover, a reversal of irradiation-induced gene expression was seen in peripheral blood and bone marrow after treatment with MSC-EV. The murine hematopoietic cell line, FDC-P1 exposed to 500cGy, showed dramatic reversal of growth inhibition and apoptosis on exposure to murine or human MSC-EV. In addition, a preparation with both exosomes and microvesicles was found to be superior to either microvesicles or exosomes alone. In addition, we have shown that both murine- and human-derived vesicles are active against irradiated murine bone marrow cells, that vesicles from fresh bone marrow cells are active, and that MSC vesicles stimulate normal murine marrow stem cell/progenitors to proliferate. These studies indicate that MSC-EV have the capacity to reverse radiation damage to bone marrow stem cells and may represent an important new strategy in radiation mitigation (45).

Engineering exosomes as therapeutics for brain disease

Though treatments for central nervous system (CNS) disorders are being rapidly developed, their delivery across the blood-brain barrier remains a significant hurdle. Exosomes can easily cross the blood-brain barrier without use of an additive vehicle (46). Indeed, a variety of administration routes successfully deliver exosomes to the CNS (46–48). This suggests that exosomes can be loaded with drugs for CNS delivery, and there is significant interest in exploiting exosomes by re-engineering them as immunomodulators or delivery platforms for cancer therapeutics (49) and vaccines (50). However, Richard Kraig and colleagues find that some exosomes are therapeutic in and of themselves.

The Kraig lab studied exosomes as a means of improving recovery from demyelination in multiple sclerosis (MS), an inflammatory disease involving oligodendrocyte loss, demyelination and failure to repair damaged myelin (46). Most current MS therapeutics reduce demyelination via immune suppression, and thus often produce harmful immune sequelae. No existing therapies treat progressive MS, and though some may aid remyelination, none extensively regenerate myelin.

The Kraig group found that exosomes derived from the serum of rats exposed to environmental enrichment (EE; volitionally increased physical, intellectual and social activity) improved recovery from lyssolecithin-induced demyelination in an *in vitro* model of MS (51). Furthermore, nasal administration of these exosomes to naïve rats increased CNS myelin, oligodendrocyte precursor and neural stem cell levels, and reduced oxidative stress. They found that these exosomes contained high levels of miR-219, a microRNA that is crucial for oligodendrocyte maturation and the formation and maintenance of compact myelin (52,53).

To mimic EE and create a scalable source of therapeutic exosomes, we stimulated primary dendritic cell cultures (DCs) with IFN γ , a pro-inflammatory cytokine that is physiologically increased during EE. Exosomes produced by IFN γ -stimulated DCs (IFN γ -DC-Exos) increased myelination and oxidative tolerance *in vitro* and *in vivo*. These IFN γ -DC-Exos also contained high concentrations of miR-219, increased oligodendrocyte precursor differentiation and improved recovery from experimental demyelination (46). Future steps toward therapeutic use of DC exosomes may include re-engineering them to contain only specific microRNAs, or altering their surface composition to target-specific cell types.

Production of extracellular vesicles in good manufacturing production

This work approaches what is necessary for the GMP production of vesicles for eventual clinical trials. The requirements include isolation approaches, quantification

of vesicles, potency determination, characterization of cell source, analysis of contents and surface epitopes of vesicles. Toxicity studies will be necessary along with evaluation of dose responses, time courses and vesicle stability (54–56). Biologic evaluations will be necessary during different phases of GMP process including production, recovery, characterization and storage. Regulations in Europe and the US will have to be rigorously followed. The regulatory considerations would take into account those for the cells of origin. In the case of MSCs, clear criteria of identity and biosafety are available. The challenge is to define criteria for EVs taking into account the following considerations. The available preparative methods result in a heterogeneous population of EVs. Further separation to a homogenous population would not be technically possible in a GMP condition that requires minimal manipulation, because there are not definite size, density or protein markers that can reliably define a specific population. Therefore, EV product should not have an absolute requirement for characterization of cell surface markers, content or for homogeneity but rather should be based on the definition and description of a size-defined (nanosize range), functionally effective population isolated using a robust, reproducible and validated process. The safety efficacy profile as well as bio-distribution needs to be demonstrated in preclinical studies. Valuation of efficacy should rely on functional potency tests relevant to the proposed application. Finally, the dose and the route of administration should be defined on the basis of efficacy and safety profiles, bioavailability and specific formulation of the therapeutic product, for example, the EV product may be cryopreserved, lyophilized, used in cell suspension or combined into a matrix.

Retroviral-like particles

There is a common consent in the field that EVs are composed of a variety of vesicles that vary in diameter, content, membrane composition and biogenesis. So far, we lack a clear understanding of the biology behind these subpopulations which results in the lack of methods to efficiently separate and study these vesicles. EV subpopulations addressed in different studies include exosomes, oncosomes, microparticles and retroviral-like particles. The latter is the focus of this section. Retroviral-like particles relate to remnants of ancestral infections by active retroviruses of germ lines in primates, which have been thereafter transmitted to the progenies (57). These sequences comprise about 8% of the human genome and are mostly silent due to methylation, recombination or mutations in either the long terminal repeats (LTRs), gag, pol, env or prt genes. Defects in either of these proteins, which are necessary for assembly of infectious viral particles, result in the production of defective retroviral particles. Thus, endogenous retroviruses can produce particles that resemble proviruses of true retroviruses

composition and, under normal conditions, can move only within cells (57). DNA hypomethylation is a common phenomenon in all cancer types, and probably due to their abundance, repetitive elements seem mostly affected by this change (58). This results in many of the endogenous retroviral sequences becoming transcriptionally active, with the increased chance of forming retroviral-like particles that can end up in the intercellular space and biofluids. In fact, retroviral-like particles have been isolated from the plasma of patients with lymphoma and breast cancer, as well as of HIV patients (59,60). In the context of EVs, several studies have reported the presence of non-coding RNA species in EVs (61), and particularly a significant enrichment of human endogenous retroviral (HERV) sequences from glioblastomas, suggesting a common biogenesis pathway between EVs and retroviral-like particles, where retroviral RNA is specifically packaged into vesicles (62,63). Furthermore, HERV sequences were shown to be transferred from normal medulloblastoma cells to human endothelial cells (HUVECs) and remained high up to 72 hours, suggesting that tumour cells can specifically package HERV RNA into particles that can be sent out to neighbouring cells to modify their phenotype and genotype. Other studies also indicate that cells produce a highly specified type of vesicles upon infection or exposure to other stimuli (64,65). Clearly, EVs share pathways with the assembly machinery of several other viruses, including HERVs, and this convergence may explain the observation of viral-like particles containing viral proteins and nucleic acid in EVs. In particular, tumour cells may be exploiting remnants of the retroviral-like packaging machinery for its own advantage to “infect” surrounding normal (or distant) cells and spread malignancy.

Extracellular vesicles in cancer

Extracellular vesicles in prostate cancer progression

As carriers of genomic and oncogenic factors, tumour-derived exosomes have been implicated in genetic exchange with non-malignant cells (66). The current evidence lends credence that exosomes are involved in communication and intercellular trafficking of bioactive molecules between tumour cells and MSCs. Abdel Mageed et al. recently showed that prostate cancer (PC) cell-derived exosomes were capable of recruiting PC patient-derived adipose stem cells to the tumour sites in vivo (67). Once engrafted, these cells underwent prostate tumour mimicry by expressing PC-specific markers. The ex-vivo treatment of patient-derived adipose stem cells by PC cell-derived exosomes triggered neoplastic reprogramming of these cells upon transplantation into immunocompromised mice. Consistent with our findings, exosomes derived from bone marrow mesenchymal stromal cells (BM-MSCs) were reported to support multiple myeloma (MM) clonal expansion and promote MM formation

in vivo (68). Ovarian and breast cancer-derived exosomes render adipose tissue-derived MSCs to undergo physical and functional characteristics suggestive of phenotypic transformation into tumour-supporting myofibroblasts in tumour stroma (69,70). In addition, cancer cells are induced to exhibit epithelial-to-mesenchymal transition (EMT) phenotype upon release of the epidermal growth factor receptor (EGFR) and tissue factor (TF)-containing exosomes (71). The TF-containing exosomes are capable of enhancing the procoagulant activity of endothelial cells – thus influencing tumour-vascular interactions (71). The final message here is that PC exosomes potentially aid aggressive epithelial cancer cells to exhibit mesenchymal characteristics and to adopt aberrant interactions with other cells at tumour sites. Conversely, in the present work, we have shown that MSC or normal PC-derived vesicles can reverse chemoresistance or anchorage-independent growth in vitro and the neoplastic phenotype in vivo in rodent models.

Although the underlying mechanisms remain elusive, tumour cell vesiculation has been implicated in cancer progression by promoting angiogenesis growth and metastasis (72,73). In addition, the tumour-associated exosomes aid cancer progression by suppressing the immune system (74,75).

Lundholm et al. (76) recently demonstrated that the expression of NKG2D ligand by PC cell-derived exosomes contributes to PC progression through downregulation of its cognate activating cytotoxicity receptor NKG2D on natural killer (NK) and CD8⁺ T cells in a dose-dependent manner. The circulating NK and CD8⁺ T cells in colorectal and prostate cancer (CRPC) patients have significantly lower levels of surface NKG2D expression, compared to healthy subjects. Interestingly, circulating exosomes from CRPC patients suppress NKG2D expression in effector lymphocytes – thus promoting tumour escape and disease progression (76). The loss frequency of cytoskeletal regulator DIAPH3 is significantly higher in advanced PC and is associated with transformation into an amoeboid phenotype marked by enhanced tumour cell migration, invasion and metastasis (77). Silencing of DIAPH3 triggers EV release by PC, which in turn activates Protein kinase B (AKT) and suppresses tumour-infiltrating immune cells (77). Collectively, the aforementioned findings attest to the emerging mechanistic role of EVs in PC progression. Targeting EV biogenesis and release by PC cells and/or their uptake by recipient cells may have preventive and therapeutic benefits for patients with advanced PC.

Extracellular vesicles in colorectal cancer

All cancers, including colorectal cancer (CRC), are characterized by driver mutations that allow their growth free of mitigating regulatory signals in their local cellular environment leading to tumour growth and metastasis. Most sporadic CRCs have mutations that activate

canonical WNT signalling, and most of these mutations are in adenomatosis polyposis coli (APC) (78). Such tumours incur activating mutations in oncogenes such as the small GTPase KRAS, mutated in 30–40% of CRC and PI3-Kinase, while other mutations occur in tumour suppressive genes like TP53 as well as TGF β pathway members like TGF β RII receptor and SMAD4. These mutational events, along with amplifications, deletions and translocations, are associated with neoplastic progression. Much of the work on such driver mutations concerns their regulation of cell autonomous effects within mutant-containing epithelial cells, but non-cell autonomous effects that pattern the tumour microenvironment can be mediated by EVs. EVs include the smaller 40–150 nm endosomally derived exosomes and the larger cell-membrane-budded microvesicles (MVs) that reach an upper limit of about 1 μ m. How oncogenic mutations in tumour cells alter EVs and how these altered EVs change the tumour microenvironment is an important new area of study. Here, we review the status of EVs in CRC. An important issue in the field is the heterogeneity of vesicles secreted from cells. It has been shown that such secreted vesicles carry both activated receptors and ligands that can signal to recipient cells, inducing cell growth and invasive properties (79) and also carry oncogenes, tumour suppressors and even RNAs that regulate tumour growth.

Two strategies have been used to address how tumour progression might alter the functional non-cell autonomous EVs output; one tests how the presence or absence of oncogenes or tumour suppressors alters the constituents of EVs; the other is to use the degree of transformation of model cells to provide a template to compare subsets of EVs. Some of the cell models used to test some of these questions are shown in Table I. EVs from different sources are added to recipient cells to test their growth-altering function. The composition and function of such EVs depends on how they are purified and sub grouped. These differences have implications for the potential biomarker properties of such EVs.

Several groups have performed comprehensive proteomic analysis of EVs from various cell line-derived (see Table I) or in vivo EVs (83,87,93). The different methods used to purify such vesicles (94) range from standard differ-

ential centrifugation alone (81,82), iodixanol [OptiPrep™] gradient and/or sucrose gradient (83,84,86,87,95), immunoaffinity isolation (86,87,96), FPLC purification (97) and flow sorting of vesicles (81,83,98). Certain core proteins are observed repeatedly using all methods, enriched to varying degrees; such enriched proteins include multiple tetraspanins like CD81 and CD9, as well as multi-vesicular body (MVB) biogenic proteins like TSG101 and ALIX (PDCD6IP) (99). Because “exosomal pellets” contain multiple other species (86), including free protein complexes (100), depending on source, lipoprotein complexes (97) and other vesicles of non-endosomal/MVB origin, whether budded directly from the plasma membrane (101) or derived from other organelles (91), the choice of purification method used in the above studies has affected which proteins are identified and influences what is termed EV-associated generally or exosome-associated specifically.

For example, LIM1863 cells, which polarize as suspended cysts in media, do not attach to plastic and produce differentiated enterocytes, and goblet cells were used to isolate distinct A33-containing or EpCAM-containing exosomes (96). Proteomic analysis of immune-captured A33 exosomes shows they contain basolateral cargo and specific trafficking proteins, many of which are basolateral trafficking proteins. EpCAM is an apical tight junction protein in LIM1863 cells; immune capture with EpCAM yielded apical proteins within the captured exosomes. These results show there are at least 2 distinct vesicle populations associated with these cells, derived potentially from 2 different cellular membranes.

Several groups have used SW480 cells derived from a primary CRC tumour and SW620 cells derived from a lymph node metastasis from the same patient to compare metastatic protein changes that occur in EVs derived from these related cell lines. These studies (83,84) show that metastatic markers are upregulated in SW620 compared to SW480 EVs, finding upregulation of MET as well as lipid raft components CAV1, FLOT1 and 2, PROM1 (CD133) and tyrosine-protein kinase Src (SRC) up in SW620 exosomes (84), while EGFR was up in SW480 exosomes. It was also found that the TNK1-RAP2a complex is unique to SW620 exosomes, which has

Table I. CRC cell lines and characteristics

DLD-1, DKO-1, DKS-8	Parental DLD-1: APC mutation KRA S +/G13D, DKs-8 [+/- with decreased transformed characteristics and DKO-1 (G13D)	(80–82)
SW480, SW620	SW480 and SW620 established from a single individuals primary cancer and lymph node metastasis, respectively. Both have a mutant KRAS (G12V) and an APC mutation	(83, 84)
LIM1863	Forms cysts in suspension with polarized cells; contains colonocytes and goblet cells	(85, 86)
LIM1215	Non-polyposis CRC cell line, MSI cancer	(85, 87–89)
HT29	Mutant APC, mutant BRAF, polarizing cell line	(90)
HCT116	β -catenin mutation and heterozygous for KRAS (G13D/ +), wild-type P53	(91)
Caco-2	Mutant APC polarizing cell line	(92)

implications for the invasive potential of such exosomes. EVs from these cells were also compared to other CRC cell lines LIM1215, HT29 as well as CRC ascites, which identified other co-expressed and different EV markers (83). In a separate study, it was shown that restoration of wild-type APC expression to SW480 cells leads to induction of the WNT inhibitor DKK4 in the cells and their exosomes, which suggests APC may also regulate WNT signalling non-cell autonomously through DKK4 secretion in exosomes.

How the presence or absence of mutant KRAS alters the proteins and function of exosomes was tested with the DLD-1 parent cell line that is heterozygous for KRAS, having one mutant activated allele and one wild-type allele. Isogenic cell lines were created from DLD-1, selectively knocking out the wild-type (DKO-1) or mutant KRAS allele (DKs-8) (78). A comprehensive proteomic analysis of these exosomes showed that, depending on cell source, they contained very different constituents, with mutant KRAS cell-derived exosomes having increased levels of SRC, EGFR and mutant KRAS compared to exosomes from wild-type KRAS DKs-8 cells (82). Functional studies showed that mutant KRAS-containing DKO-1 exosomes could transfer the mutant KRAS protein to wild-type KRAS-expressing cells, thereby demonstrating non-cell autonomous transfer of an oncogene. Such mutant KRAS exosomes could also induce cell growth and invasive characteristics in these recipient cells. It was also shown that DKs-8 exosomes contained many more RNA binding proteins than those derived from DKO-1 cells, which has implications for these exosomes as RNA carriers.

It was shown that exosomes released by mutant KRAS cells contain increased levels of the EGFR ligand, amphiregulin (AREG) (81). EGFR ligands are transmembrane proteins that can act as secreted factors when they are cleaved from the cell surface via metalloproteases; it was demonstrated that EGFR ligands AREG, transforming growth factor alpha (TGFA) and heparin binding EGF like growth factor (HBEGF) are maintained as intact transmembrane proteins within exosomes (81). In separate experiments, individual EGFR ligands were tested for their ability to induce invasive characteristics in recipient cells (81). It was shown that exosomal AREG significantly enhanced the invasive capacity of recipient breast cancer cells compared to secreted soluble AREG or other EGFR ligands, whether these ligands were in exosomes or soluble. The invasive effect of AREG exosomes was, at least in part, due to recipient cell EGFR. Using fluorescence-activated vesicle sorting (FAVS), a flow cytometric method for analyzing and purifying exosomes (81,98,101), it was shown that DLD-1 cells secreted a complex mixture of exosomes that carried varying levels of the EGFR ligands HBEGF, AREG and TGFA that fell into at least 9 classes of distinct exosomes.

These results show that the exosomal composition from these cells is complex and that proteomic analysis of such exosomes identifies proteins in multiple subgroups of exosomes present in these preparations.

In other work using HCT-116 cells, mutant KRAS was shown to induce P53 secretion from a clearly non-exosomal vesicular component (91). Here, P53 is secreted from mutant KRAS-expressing cells through binding to the EMT-associated transcription factor SNAIL (which is co-secreted) and a P53 nuclear export sequence mutation does not inhibit this process, suggesting that such P53 export is derived from a nuclear subcompartment. Consistent with this finding, P53 vesicular structures were surrounded by nuclear membrane component, and export occurs through a vesicle-like transport mechanism requiring cytoskeleton function. Mutant KRAS induces CAV1, and results suggest CAV1 regulated endocytosis is responsible for P53 uptake from the media. Autoantibodies to SNAIL were found in cancer patient sera, which may be a potential biomarker for CRC. These results show complex functions for mutant KRAS-induced changes in secreted factors, leading to the export and uptake of the P53 tumour suppressor and the EMT inducer, SNAIL. The pace of cancer related findings continues to accelerate.

EVs in cardiovascular diseases

Functional role for EVs in the cardiovascular system

Cardiomyocytes (CMs), cardiac fibroblasts, endothelial and cardiac progenitor cells have all been shown to secrete EVs (102,103). Like EVs from other cell types, they contain a variety of proteins and RNAs from the parent cell and appear to have functional effects on neighbouring cells.

CM- and fibroblast-derived EVs

EV release from CMs was first reported by Gupta and Knowlton in primary cultures of adult rat CMs, and appeared to be regulated by hypoxia (104). In a second study, hypoxia led to the release of the 26 kDa transmembrane form of TNF- α in CD63⁺ EVs. Exposure of healthy CMs to these EVs induced apoptosis, suggesting that stress may lead to deleterious signalling between CMs (105). A recent study also demonstrated the transfer of DNA and RNA from CMs to fibroblasts (102). EVs released by HL-1 cells were found to contain DNA and RNA, as labelled with acridine orange, and when added to NIH-3T3 cultures, could transfer the acridine orange staining into NIH-3T3 fibroblasts nuclei, thereby demonstrating EV-mediated transfer of genetic information from CMs to fibroblasts. Cargo present within the EVs included ribosomal RNA and mRNA coding for proteins, and transfer of this produced changes in gene expression within the fibroblasts. Our own recent work demonstrates

clear uptake of EVs derived from primary neonatal rat CM cultures into cardiac fibroblasts (Fig. 1), suggesting that EVs may indeed be an important mode of paracrine signalling among cells of the cardiovascular system. Interestingly, a recent study demonstrated that cardiac fibroblast-derived EVs also transfer information to CMs (103). Cardiac fibroblast-derived EVs contained many passenger strand miRNAs that are normally subjected to intracellular degradation. These miRNAs were shown to be potent pro-hypertrophic factors in the CMs, and inhibition of one of these, miR-21-3p, attenuated pathology (Fig. 1).

In the present work, using a unique mouse model of genetic fluorescent labelling of different cell types, the Das laboratory has been assessing the contribution of circulating EVs in murine models of heart disease. The profile and content of EVs in human heart disease is being simultaneously addressed. Functionality of EVs derived from the heart: using EVs isolated from human heart failure patients, control patients or post-myocardial infarction patients, the Das laboratory is assessing their role in altering gene expression and function in human iPSC-derived CMs and fibroblasts. Together these experiments are expected to provide a more detailed characterization of the origin and function of heart-derived EVs.

Conclusions

This represents work by individuals in initiative 4 on clinical utility of exRNAs as therapeutic agents and developing scalable technologies required for these studies within the NIH Extracellular RNA Communication Program. It also includes selected work by collaborators. The work on plant EVs is quite unique and represents an area of investigation which will be rapidly expanding and could be a major factor in eventual clinical applications. The studies on repair of renal, bone marrow, cardiac, pulmonary and neural tissues highlight the whole area of

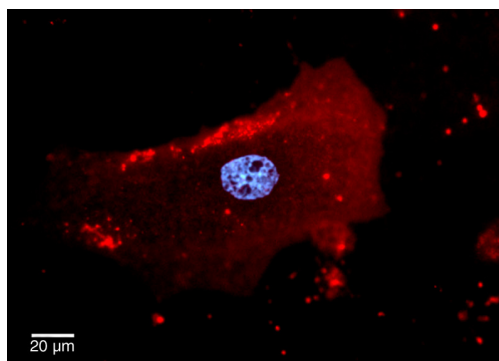


Fig. 1. CMs stained with CellTracker™ Red were washed and cultured in serum free media for 48 hours. EVs were isolated from media using ultracentrifugation and visualized being taken up by cardiac fibroblasts using an Olympus BX62 microscope with Qimaging EMc2 EMCCD cooled camera (red, cell tracker red for membrane; blue, hoechst 33,342 for nucleus).

vesicle potential in regenerative medicine, which continues to expand. The role of retroviral-like particles in EVs is of intense interest and raises some concern. The role of EVs in cancer is also of intense interest. Here, there is evidence for “good” and “bad” vesicles. The “good” may be derived from normal tissue or mesenchymal stem cells and can reverse malignant phenotypes while the “bad” derive from neoplastic cells and can progress a malignant phenotype. In a similar fashion, there are both “good” and “bad” vesicles in pulmonary and cardiovascular diseases. These observations illustrate the basic heterogeneity of EVs from different tissues under different separative approaches and subjected to various stimuli. Further, heterogeneity rests in the nature and condition of vesicle target cells. This heterogeneity is a critical consideration as to eventual production of EVs in good manufacturing production. The field is moving rapidly toward a variety of therapeutic strategies with clinical protocols already evolving.

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